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Two *Anabaena* sp. Strain PCC 7120 DNA-Binding Factors Interact with Vegetative Cell- and Heterocyst-Specific Genes

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The DNA-binding factor BifA (previously called VF1) binds upstream of the developmentally regulated site-specific recombinase gene *xisA* in the cyanobacterium *Anabaena* sp. strain PCC 7120. Besides binding *xisA*, BifA also binds the *glnA*, *rbcL*, and *nifH* promoter regions. DNase I footprint analysis of BifA binding to *glnA* showed a protected region −125 to −148 bp upstream of the translation start site. The binding site is between the major *glnA* transcription start site used in vegetative cells (RNA_{II}) and the major transcription start site used under nitrogen-deficient conditions (RNA_{I}). The two BifA-binding sites on the *rbcL* promoter were localized to a 24-bp region from +12 to −12 nucleotides and to a 12-bp region from −43 to −54 nucleotides with respect to the transcription start site. Comparison of the BifA binding sites on the *glnA*, *xisA*, and *rbcL* upstream regions revealed the consensus recognition sequence TGT($N_{9\ or\ 10}$) ACA. We have identified a second DNA-binding activity (factor 2) that interacts with *rbcL* and *xisA* upstream regions. Factor 2 can be resolved from BifA by heparin-Sepharose chromatography and was present in a *bifA* mutant. Analysis of partially purified vegetative cell and heterocyst extracts showed that whereas BifA was present in both cell types, factor 2 was present only in vegetative cells. DNase I footprint analysis of factor 2 binding to *rbcL* showed protection of a 63-bp region between positions −15 and −77 with respect to the transcription start site. The factor 2 binding site on *xisA* was localized to a 68-bp region that showed considerable overlap with the BifA binding sites.

Anabaena sp. strain PCC 7120 is a filamentous cyanobacterium that is capable of biological nitrogen fixation, a process that involves the reduction of dinitrogen to ammonia by oxygen-labile nitrogenase. The problem of fixing nitrogen while simultaneously producing oxygen from photosynthesis is circumvented by compartmentalization of nitrogenase in heterocysts. Heterocysts are highly specialized cells that differentiate at fairly regular intervals along each filament in response to deprivation of combined nitrogen. The differentiation of a vegetative cell into a heterocyst is a complex process that involves a number of morphological and biochemical changes and is regulated by both external cues and intercellular communication. The heterocyst itself is terminally differentiated, lacks the oxygen-evolving photosystem II, and has a unique morphology and biochemistry that provides the anaerobic microenvironment necessary for nitrogen fixation (1, 21, 40).

Heterocyst development requires global changes in gene expression, with some genes, such as the *rbcLS* operon, which encodes the large and small subunits of ribulose-1,5-bisphosphate carboxylase, being turned off and genes involved in nitrogen fixation, such as the *nifHDK* operon, which encodes the nitrogenase subunits, being turned on (16, 18, 22). In contrast, genes such as *glnA*, which encodes glutamine synthetase, are expressed in both vegetative cells and heterocysts (35). Promoter fusions to luciferase reporter genes have demonstrated that transcription of *rbcLS* is confined to vegetative cells, while transcription of *nifHDK* is localized to heterocysts (13).

Differential gene regulation in vegetative cells and heterocysts is probably caused by different RNA polymerase sigma factors in concert with other *trans*-acting factors. Developmental-stage- or cell-specific regulation by sigma factors has been shown to exist in *Bacillus* (26) and *Streptomyces* (10) spp. In PCC 7120 three sigma factors have been found so far. One of them, *sigA*, is expressed in both the presence and absence of combined nitrogen, and the other two, *sigB* and *sigC*, are expressed transiently under nitrogen-limiting conditions (6, 7). Besides specialized sigma factors, changes in gene expression can be caused by *trans*-acting DNA-binding proteins (12). A number of DNA-binding proteins in cyanobacteria have been described recently. PepB, a phycoerythrin promoter-binding protein from *Fremyella diplosiphon*, has been partially characterized (33). RcaA and RcaB are two DNA-binding proteins that have been shown to interact with the promoter region of the *cpeBA* operon in *Calothrix* sp. strain PCC 7601 (34). A DNA-binding protein that binds upstream of the light-regulated *psbDII* gene in *Synechococcus* sp. strain PCC 7942 has been partially characterized (9). Two regulatory genes, *cysR* and *ntcA*, from *Synechococcus* sp. strain PCC 7942 have been sequenced and are proposed to encode DNA-binding proteins. CysR, which regulates sulfate transport (25), and NtcA, which regulates nitrogen assimilation (14, 36, 37), both belong to a family of prokaryotic regulatory proteins represented by the cyclic AMP receptor protein (CRP).

The differentiation of a PCC 7120 vegetative cell into a heterocyst is associated with two DNA rearrangements, one of which involves the excision of an 11-kb DNA element from the coding region of the *nifD* gene (17). This rearrangement results in the formation of a complete *nifD* coding sequence and allows transcription of the *nifHDK* operon from a promoter upstream of *nifH* (18). The *xisA* gene, located at one end of the *nifD* element, encodes the site-specific recombinase responsible for excision of the element from the chromosome during heterocyst differentiation (8, 19, 24).

Initial attempts to express *xisA* in vegetative cells by using a shuttle vector failed, although the same constructs could be expressed in *Escherichia coli*. This suggested the presence of a

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regulatory region upstream of the translation start that prevents expression of the *xisA* gene in vegetative cells. Deletion of this region led to the expression of *xisA* in vegetative cells, as evidenced by the excision of the *nifD* element from the chromosome (8). The DNA-binding factor BifA (binding factor A, formerly VF1) was shown to bind the *xisA* upstream region in proximity to the sequences involved in repression of *xisA* expression in vegetative cells. DNase I footprinting and deletion analysis of the *xisA* upstream region mapped the BifA binding site to a 54-bp region upstream of the open reading frame (11). We have recently cloned the *bifA* gene, and like NtcA and CysR, its protein product is similar to CRP (39). BifA is most similar to NtcA (77% identical) and may be the *Anabaena* homolog of the *Synechococcus* NtcA protein.

We have further characterized the binding of BifA to the *Anabaena* sp. strain PCC 7120 *glnA*, *rbcL*, and *nifH* upstream regions and have determined a consensus BifA binding sequence. Furthermore, we demonstrate the presence of a second DNA-binding activity (factor 2) in vegetative cells that interacts with the *rbcL* and *xisA* upstream regions in vitro.

MATERIALS AND METHODS

Strains and culture conditions. *Anabaena* sp. strain PCC 7120 was grown in 100-ml BG-11 liquid cultures. Larger-scale (2- to 8-liter) cultures were grown in the liquid medium of Allen and Arnon diluted eightfold (AA/8) with some modifications. The growth conditions and the compositions of the media have been described previously (18).

E. coli strains were maintained in LB liquid or on LB agar medium (Lennox L). For plasmid preparation, strains were grown in 0.5× TB liquid medium, a variation of Terrific Broth, as described previously (8). Media were supplemented with appropriate antibiotics according to standard protocols (32). Plasmids were maintained in *E. coli* DH5α-MCR or DH10B.

Partial purification of DNA-binding factors. Vegetative cell filaments from 2- or 8-liter cultures grown to an optical density of 0.3 at 750 nm were harvested by centrifugation at 4,500 × *g* for 5 min. The pelleted cells (in approximately 10 ml of medium) were suspended with 20 ml of homogenization medium (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 2 mM dithiothreitol, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol) and homogenized with an equal volume of glass beads (0.10-mm diameter) for 3 min at 4°C in a homogenizing mill (B. Braun Biotech Intl., Allentown, Pa.) (11). Alternatively, the cells were broken by using a cell disruptor with an air-driven pump (Stansted Fluid Power Limited, Stansted, Essex, England). The cells from a 2- or 8-liter culture were suspended in 100 ml of cold (4°C) homogenizing medium and passed through the Stansted cell disruptor twice at 12,000 lb/in^2. This method consistently gave over 99% cell breakage. For heterocyst extracts, the vegetative cells were first disrupted by passing induced filaments through the Stansted cell disruptor twice at 12,000 lb/in^2. The intact heterocysts were collected as a pellet, following centrifugation at 4,500 × *g* for 5 min. The heterocyst pellet was washed once with homogenizing medium, suspended in 10 ml of homogenizing medium, and then passed through the Stansted cell disruptor twice at 20,000 lb/in^2. This method consistently gave over 90% breakage of the heterocysts. The cell lysate was clarified at 31,000 × *g* for 20 min and 142,000 × *g* for 60 min. The supernatant was loaded onto a 3-ml heparin-Sepharose CL6B (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.) column, equilibrated with 0.1 M ammonium sulfate in buffer A (50 mM Tris-HCl [pH 7.5], 0.1 mM EDTA, 0.1% Triton X-100, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol). The column was washed with 100 ml of 0.1 M ammonium sulfate in buffer A and eluted with a 50-ml linear gradient of 0.1 to 0.5 M ammonium sulfate in buffer A (11). A gradient of 0.1 to 1.0 M ammonium sulfate in buffer A was used in initial experiments. Two-milliliter fractions were collected and dialyzed against 2.5 liters of 50 mM Tris-HCl (pH 7.5)–0.1 mM EDTA–2 mM dithiothreitol–0.5 mM phenylmethylsulfonyl fluoride–10% glycerol. All steps, including homogenization, were carried out at 4°C. The dye binding assay of Bradford (5) was used to assay protein concentration.

Mobility shift assay. Mobility shift assays were performed as described by Ausubel et al. (2) and included the modifications described by Chastain et al. (11). A 5-μl sample of heparin-Sepharose-fractionated protein was equilibrated with 0.2 to 0.5 ng of labeled DNA fragment in binding buffer with or without 5 mM $MgCl_2$, in a final volume of 20 μl. The binding buffer contained 4 mM Tris-HCl (pH 8.0), 12 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.9), 12% glycerol, 60 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, and 1.0 μg of poly(dI-dC) (Pharmacia LKB Biotechnology Inc.). After 20 min at 24°C, the samples were loaded onto a 5% polyacrylamide gel (20 by 15 cm; acrylamide/bisacrylamide, 30:1; 50 mM Tris, 380 mM glycine, 2 mM EDTA, 2.5% glycerol). The samples were electrophoresed for 2.5 h at 30 mA at 4°C in Tris-glycine buffer (50 mM Tris [pH 8.5], 100 mM glycine, 2 mM EDTA). Gels were dried and exposed to X-ray film. The factor 2-*xisA* complex was run on polyacrylamide gels (10 by 7.5 cm) for 30 min at 200 V.

Preparation of DNA fragments for mobility shift assays. DNA fragments for mobility shift assays and DNase I footprinting were labeled with [α-^{32}P]dATP or [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I by standard procedures (2). The nucleotide positions referred to in this paper are with respect to the translation start site designated as +1, unless mentioned otherwise, since the *xisA* promoter has not been mapped and *glnA* is known to have multiple transcription start sites.

Plasmids pAM247, pAM251, and pAM249 contain the *xisA* gene with progressively increasing deletions of the upstream sequences. They correspond to plasmids pAM262, pAM265, and pAM264, respectively, except that the vector is pBluescript KS(+) (Stratagene, La Jolla, Calif.) rather than pRL191*tac* (8). Fragments from pAM247, pAM251, and pAM249 containing 335, 235, and 206 bp of *xisA* upstream region, respectively, were released for labeling by digestion with *Xba*I and *Sca*I and carried an additional 24 bp of vector sequence. The *Xba*I-to-*Sca*I fragment of pAM251 was cloned into the *Xba*I-*Sma*I site of pBluescript KS(−) to yield pAM709. A 281-bp fragment containing 235 bp of *xisA* upstream region was released from pAM709 by digestion with *Xba*I and *Hin*dIII for mobility shift assays.

A 504-bp *Alu*I fragment from pAn154.3 containing the *nifH* gene promoter (nucleotides −573 to −70 relative to the translation start site) (22) was subcloned into the *Eco*RV site of pBluescript KS(+) to produce pAM361. A *Tha*I-*Hin*dIII fragment of pAM361, which contains the *nifH* upstream region from −260 to −70, was cloned into the *Eco*RV-*Hin*dIII site of pBluescript KS(+) to produce pAM657. The 191-bp *nifH* upstream region was obtained from pAM657 as a 233-bp *Xba*I-*Hin*dIII fragment.

In initial experiments involving the *glnA* promoter, a 432-bp *Hin*dIII-*Xba*I fragment from plasmid pCP106 (11) containing 410 bp of *glnA* upstream region (nucleotides −381 to +29) was used in mobility shift assays. *Hin*cII digestion of this fragment released a 161-bp *Hin*dIII-*Hin*cII fragment and a 271-bp *Hin*cII-*Xba*I fragment. The 271-bp *Hin*cII-*Xba*I frag-

ment containing 249 bp of the *glnA* promoter region (nucleotides −220 to +29) was subcloned into the *Xba*I-*Sma*I site of pBluescript KS(+) to produce pAM658. For subsequent experiments, a 292-bp *glnA* fragment was released from pAM658 by digestion with *Xba*I and *Hin*dIII.

A 396-bp *Hpa*I fragment from pAn602 (30) containing the upstream region of the *rbcL* gene (nucleotides −600 to −205) was subcloned into the *Eco*RV site of pBluescript KS(+) to produce pAM496. A 440-bp fragment was released from pAM496 by digestion with *Xba*I and *Hin*dIII, labeled, and then digested with *Hin*fI to produce a 315-bp *Xba*I-*Hin*fI fragment containing 275 bp of the *rbcL* upstream region (AM496Δ1). AM496Δ104 is a 94-bp fragment that was generated by exonuclease III deletion of pAM496. pAM496 was cut with *Hin*dIII and *Apa*I, and exonuclease III digestion and isolation of deletion clones were done according to the vendor's instructions (Erase-a-Base kit; Promega, Madison, Wis.). Fragments for mobility shift assays were released from the deletion plasmids by digestion with *Kpn*I and *Eco*RI.

DNA sequencing. Sequencing from double-stranded DNA was performed with a Sequenase kit (United States Biochemical Corporation, Cleveland, Ohio) and [α-^{32}P]dATP. Plasmid DNA was prepared by an alkaline lysis-polyethylene glycol precipitation procedure (23). For the footprinting gels, a DNA sequence ladder was prepared on the basis of the chemical cleavage method of Maxam and Gilbert (2).

DNase I footprinting. The lower, antisense, strands of DNA fragments were labeled by using the Klenow fragment of DNA polymerase I for DNase I footprinting experiments. For BifA, an *Sst*II-*Hin*dIII fragment from pAM658 containing 249 bp of *glnA* upstream region (25,000 cpm/0.77 ng) and an *Xba*I-*Hin*fI fragment of pAM496 containing 275 bp of *rbcL* upstream region (10,000 cpm/0.38 ng) were used. For factor 2, an *Xba*I-*Kpn*I fragment of p496Δ104 containing 94 bp of the *rbcL* upstream region (10,000 cpm/0.13 ng) and an *Xba*I-*Sca*I fragment of pAM247 containing 335 bp of the *xisA* upstream region (10,000 cpm/0.43 ng) were used. Approximately 10,000 cpm of labeled fragment (25,000 cpm for *glnA*) was incubated with different amounts of heparin-Sepharose-fractionated BifA or factor 2 in a 20-μl reaction volume containing 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 6% glycerol, and 1 μg of poly(dI-dC). BifA and factor 2 were obtained from peak heparin-Sepharose fractions that clearly resolved factor 2 from BifA. The amount of peak fraction required to shift all of the probe fragment into the expected complex was determined by mobility shift assays. After a 20-min incubation at 24°C, 1 μl of 23-U/ml DNase I (Sigma Chemical Co., St. Louis, Mo.) in 25 mM Tris-HCl (pH 7.5)–25% glycerol was added. DNase I digestion was terminated after 2 min with 25 μl of stop solution containing 0.3 M sodium acetate (pH 5.2), 25 μg of tRNA, and 80 mM EDTA. The DNA was precipitated with 125 μl of ethanol, and the dry pellet was taken up with 10 μl of 1:3-diluted sucrose-urea loading dye (300 mg of sucrose, 840 mg of urea, 40 μl of 0.5 M EDTA, 100 μl of 0.1% bromophenol blue, and 100 μl of 0.1% xylene cyanol). The samples were heated to 90°C for 3 min and loaded onto 6% sequencing gels.

In reactions involving factor 2, $MgCl_2$ was left out of the binding reaction mixture but instead was added to the DNase I solution, since nucleases present in the heparin-Sepharose fractions caused degradation of the DNA probe.

RESULTS

A second DNA-binding activity in vegetative cells. Our initial experiments with partially purified *Anabaena* sp. strain PCC 7120 vegetative cell protein extracts showed the presence of a DNA-binding factor, BifA, which had specific affinity for the upstream region of the *xisA* gene. Mobility shift assays and competition experiments showed that BifA also binds to *glnA*, *rbcL*, and *nifH* upstream regions (11). Our further characterization of BifA and its interaction with these promoters has identified the presence of an additional DNA-binding activity in vegetative cell extracts that interacts with the *xisA* and *rbcL* upstream regions.

A vegetative cell lysate was fractionated by heparin-Sepharose column chromatography, and samples from a 0.1 to 1.0 M ammonium sulfate elution gradient were assayed for electrophoretic mobility shift with DNA fragments from the upstream regions of the *glnA*, *nifH*, and *rbcL* genes (Fig. 1). The *glnA* and *nifH* promoter fragments showed a single complex with peak binding around fraction 14 (Fig. 1). The binding profile matches the elution profile of BifA as observed by using the

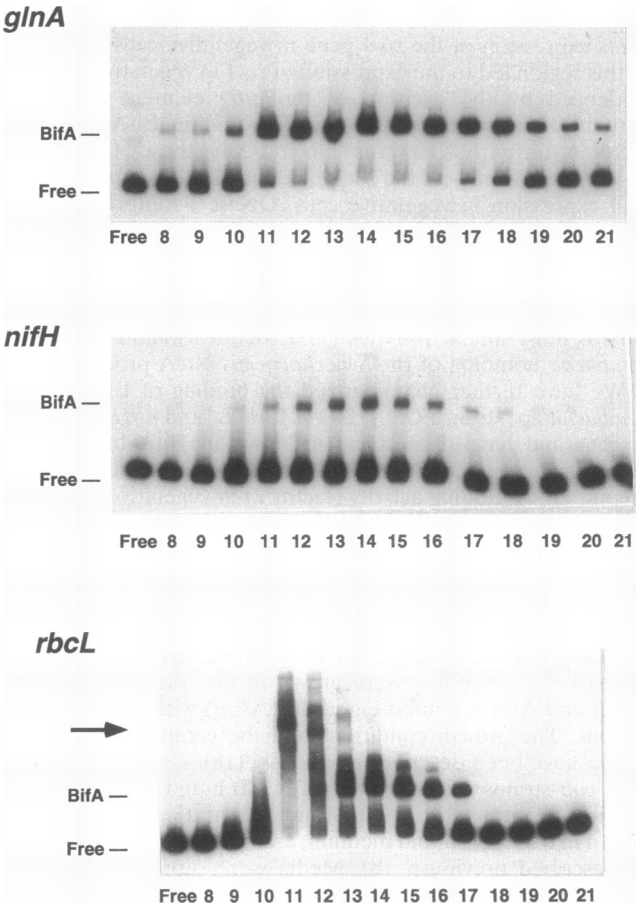

FIG. 1. BifA interacts with the *glnA*, *nifH*, and *rbcL* upstream regions. Mobility shift assays of a PCC 7120 vegetative cell lysate fractionated by heparin-Sepharose chromatography are shown. Samples (5 μl) from column fractions collected during elution with a linear 0.1 to 1.0 M ammonium sulfate gradient were equilibrated with 15,000 cpm of labeled DNA fragment and analyzed on a 5% polyacrylamide gel. The labeled DNA probes were as follows: *glnA*, *Xba*I-*Hin*dIII fragment of pAM658 containing 249 bp of *glnA* upstream region; *nifH*, *Xba*I-*Hin*dIII fragment of pAM657 containing 191 bp of *nifH* upstream region; *rbcL*, *Xba*I-*Hin*fI fragment of AM496Δ1 containing 275 bp of *rbcL* upstream region. The positions of free DNA probe and the BifA-DNA complex are shown. The arrow marks the position of a second DNA-binding activity observed with the *rbcL* probe. Numbers refer to the heparin-Sepharose column fractions.

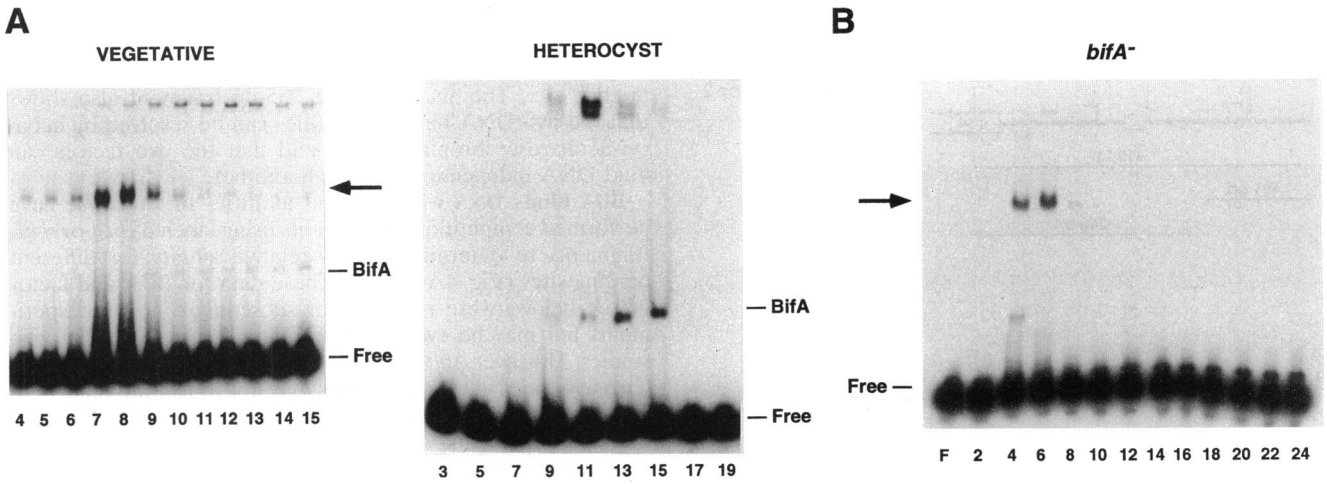

FIG. 2. A second DNA-binding activity interacts with the *rbcL* upstream region. Mobility shift assays were performed with protein extracts from wild-type vegetative cells or heterocysts (A) and from vegetative cells of a *bifA* mutant strain (B). Samples from heparin-Sepharose column fractions collected during elution with a linear 0.1 to 0.5 M ammonium sulfate gradient were equilibrated with a labeled *Eco*RI-*Kpn*I fragment from pAM496Δ104 containing 94 bp of the *rbcL* upstream region and analyzed by mobility shift assay on a 5% polyacrylamide gel. The positions of free DNA probe and the BifA-DNA complex are shown. The arrow marks the position of the second DNA-binding activity. Numbers refer to the heparin-Sepharose column fractions. F, free DNA probe.

xisA promoter fragment under similar conditions (11). The complex formed with *nifH* shifted a significantly smaller proportion of the labeled fragment than those with the other probes, indicating a weaker binding affinity under these conditions.

A second DNA-binding activity was detected when the *rbcL* promoter fragment was used as the probe. The *rbcL* promoter fragment showed two peaks of binding activity in the heparin-Sepharose protein fractions (Fig. 1). The later-eluting peak (fractions 12 to 17) matched the elution profile of BifA. An earlier-eluting DNA-binding activity was present in fractions 11 and 12 and showed shifted bands with lesser mobility than the BifA complex. This second DNA-binding activity was also detected in mobility shift assays using an *xisA* probe (see below).

In these initial studies, the peak column fractions that contained the second binding activity also contained some BifA. For all further experiments, proteins from the heparin-Sepharose column were eluted by using a shallower ammonium sulfate gradient (0.1 to 0.5 M) that resolved the second activity from BifA (see Fig. 2 and 3). For convenience, the component(s) that produces the earlier-eluting DNA-binding activity with *rbcL* and *xisA* will be referred to as factor 2.

DNA-binding activities in extracts from vegetative cells and heterocysts. Analysis of DNA-binding proteins in extracts from vegetative cells and heterocysts showed that whereas BifA DNA-binding activity is present in both cell types, factor 2 is present only in vegetative cells (Fig. 2A). Vegetative cell and heterocyst lysates were fractionated by heparin-Sepharose column chromatography and assayed by electrophoretic mobility shift with the *rbcL* fragment AM496Δ104. Both extracts contain BifA and an uncharacterized upper band. Factor 2 binding activity peaks in vegetative cell fractions 7 and 8 but is absent in similar fractions of the heterocyst extract. The apparent low level of BifA activity is due to the AM496Δ104 probe, which interacts well with factor 2 but does not bind strongly to BifA (see Fig. 3). A *glnA* probe, which interacts with only BifA, showed strong BifA DNA-binding activity in both vegetative cells and heterocysts (data not shown).

Factor 2 DNA-binding activity in a *bifA* mutant strain. Heparin-Sepharose-purified protein extracts from a *bifA* mutant of PCC 7120 (38), which do not contain any BifA DNA-binding activity, show the presence of factor 2 DNA-binding activity (Fig. 2B). Factor 2 DNA-binding activity was present in fractions 4 to 8. BifA DNA-binding activity, which typically peaks around fractions 14 to 16, was not detected in this strain. These results demonstrate that factor 2 is distinct from BifA and that its interaction with the *rbcL* upstream region is independent of BifA.

Localization of the *glnA*, *rbcL*, and *xisA* binding sites. DNA fragments from the *glnA*, *rbcL*, and *xisA* upstream regions were tested in mobility shift assays for binding to heparin-Sepharose-purified protein fractions that contained either BifA or factor 2 to localize their binding sites (Fig. 3). The interaction of BifA with the *glnA* upstream region was initially identified by using a 410-bp DNA fragment that included the region from position −381 to +29 with respect to the *glnA* translation start site (Fig. 3A). A *Hin*cII site was used to localize the BifA binding site to a 249-bp fragment that included the region from −220 to +29 with respect to the translation start site. None of the *glnA* fragments showed any detectable interaction with factor 2.

Binding of both BifA and factor 2 was localized to a 275-bp region of *rbcL* that contains the transcription start site (Fig. 3B; see Fig. 1). A 94-bp fragment (AM496Δ104, which was generated by exonuclease III digestion of pAM496) that showed weak binding to BifA but strong binding to factor 2 was identified (Fig. 2 and 3B). The results with this fragment show that the BifA and factor 2 binding sites in the *rbcL* upstream region can be resolved and that factor 2 binding does not require the presence of the BifA binding site.

Initial assays showed that both BifA and factor 2 interacted with a DNA fragment containing 335 bp of the *xisA* upstream region (Fig. 3C). A 235-bp fragment, which contains all three BifA binding sites (Fig. 3C, fraction 15), also forms a complex with factor 2 (Fig. 3C, fraction 11). However, a 206-bp *xisA* fragment that lacks one of the three BifA binding sites (Fig. 3C, fraction 15), failed to form a complex with factor 2 (Fig. 3C, fraction 11). These mobility shift assays show that although the BifA and factor 2 binding sites are close to each other in

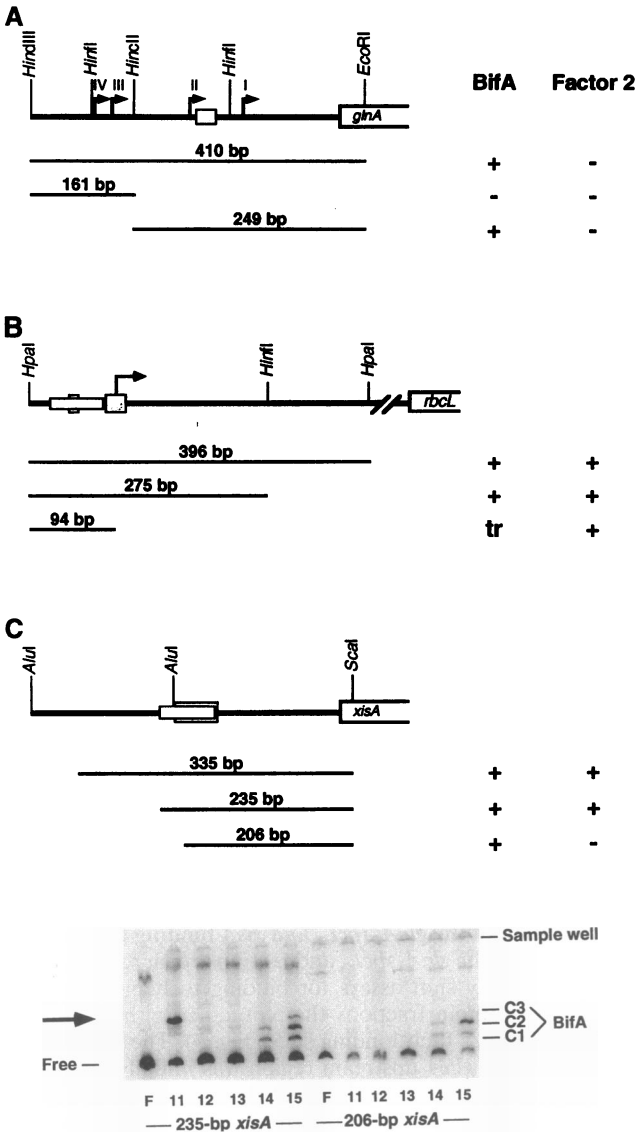

FIG. 3. Localization of the BifA and factor 2 binding sites in the *glnA*, *rbcL*, and *xisA* upstream regions. For each gene the map shows the open reading frame extending to the right, with arrows marking transcription start sites. BifA and factor 2 binding sites are represented as gray and open boxes, respectively (see Fig. 5 and 7). Shown below each map are DNA fragments that were used in mobility shift assays. The corresponding DNA-binding activities are shown to the right. DNA binding was determined by mobility shift assays using heparin-Sepharose fractions containing either BifA or factor 2. tr, trace binding. (A) *glnA* upstream region. The four *glnA* transcripts, I to IV, are shown (35). (B) *rbcL* upstream region. (C) *xisA* upstream region. The BifA binding site on *xisA* were previously determined (11). The mobility shift assay shows the interaction between factor 2 and the *xisA* upstream region. The labeled 235-bp fragment (nucleotides +12 to −222) (10,000 cpm) or the labeled 206-bp fragment (nucleotides +12 to −192) (10,000 cpm) was incubated with 4 μl of heparin-Sepharose-purified protein extract, and the DNA-protein complexes were analyzed by mobility shift assays on 5% polyacrylamide gels (10 by 7.5 cm) run for 30 min at 200 V. The positions of free DNA probe and the three BifA-DNA complexes (C1 to C3) are shown. The arrow marks the position of the factor 2-DNA complex. The uppermost band in each lane marks the position of the sample well. The low-mobility band present in each lane is a contaminant of the labeled probe. F, free DNA probe; 11 to 15, heparin-Sepharose column fractions collected during elution with a linear 0.1 to 0.5 M ammonium sulfate gradient.

the *xisA* upstream region, they can be partially resolved and that BifA's interaction with at least its two proximal binding sites does not require the presence of a complete factor 2 binding site. The assay with the 235-bp fragment also shows that the two DNA-binding activities can be resolved by heparin-Sepharose chromatography and that the two factors can bind DNA independently of each another.

BifA binds DNA with different affinities in vitro. We have performed competition experiments using labeled *glnA* or *rbcL* fragments to determine BifA's relative affinity for different binding sites (Fig. 4A and B). These data for BifA and factor 2 (see below) were required for subsequent in vitro experiments but may be eventually correlated to results of in vivo studies. However, the relative binding affinities determined in vitro with linear DNA fragments may not reflect the relative in vivo regulatory activities. A 10-fold excess of unlabeled *glnA* fragment significantly reduced the amount of labeled BifA-*glnA* complex (Fig. 4A). A 50-fold excess of unlabeled *xisA* fragment was required to achieve a similar reduction of the labeled BifA-*glnA* complex. While a 50-fold excess of unlabeled *rbcL* fragment reduced the amount of labeled BifA-*glnA* complex formed by about one-half, a 50-fold excess of *nifH* fragment did not significantly interfere with the formation of BifA-*glnA* complex. A similar gradation in the affinities of the various promoter fragments for BifA was observed when a labeled *rbcL* fragment was used (Fig. 4B). A 25-fold excess of unlabeled *glnA* fragment and a 50-fold excess of *xisA* fragment eliminated the binding of labeled *rbcL* to BifA. A 50-fold excess of unlabeled *rbcL* only partially reduced BifA binding, while a 200-fold excess of *nifH* fragment showed only a slight effect on the binding. Although the interaction between BifA and *nifH* is weak, we believe that it is specific. Mobility shift assays of column fractions showed a typical BifA elution profile and a distinct shifted band (Fig. 1). Also, an unlabeled *xisA* fragment can compete with the binding of BifA to a labeled *nifH* fragment (11). Taken together, the competition experiments show that BifA binds to the four promoter fragments in vitro, with an affinity in the order of *glnA* > *xisA* > *rbcL* >> *nifH*.

Factor 2 has greater affinity for *rbcL* than for *xisA* in vitro. Competition experiments using a labeled *rbcL* fragment showed that factor 2 binding was specific for *rbcL* and *xisA* and that factor 2 binding was strongest for *rbcL* (Fig. 4C). A 25-fold excess of the unlabeled *rbcL* fragment nearly eliminated factor 2 binding to a labeled *rbcL* fragment, while a 50-fold excess of unlabeled *xisA* fragment caused a lesser reduction in binding. A 50-fold excess of a *nifH* fragment, a *glnA* fragment, or a 35-bp oligonucleotide containing the BifA binding site on *glnA* did not significantly affect the binding of labeled *rbcL* to factor 2 (Fig. 4C).

BifA binding sites on *glnA* and *rbcL*. DNase I footprinting and deletion analysis of the *xisA* upstream region mapped three BifA binding sites to a 54-bp region upstream of the open reading frame (11). The region of the *glnA* promoter that interacts with BifA was identified by DNase I footprinting and mapped to a region from −125 to −148 bp upstream of the translation start site (Fig. 5A). This protected region is between the two major *glnA* transcription start sites and extends from 7 nucleotides downstream of the *E. coli*-type promoter start site (RNA_{II}) to 35 nucleotides upstream of the *nif*-type promoter start site (RNA_I) (35). The labeled antisense strand showed a DNase I-hypersensitive site at −130.

DNase I footprinting of the *rbcL* promoter region with BifA showed the presence of two BifA binding sites (Fig. 5A). These sites are at −493 to −516 bp and at −547 to −558 bp upstream of the translation start site. The proximal 24-bp

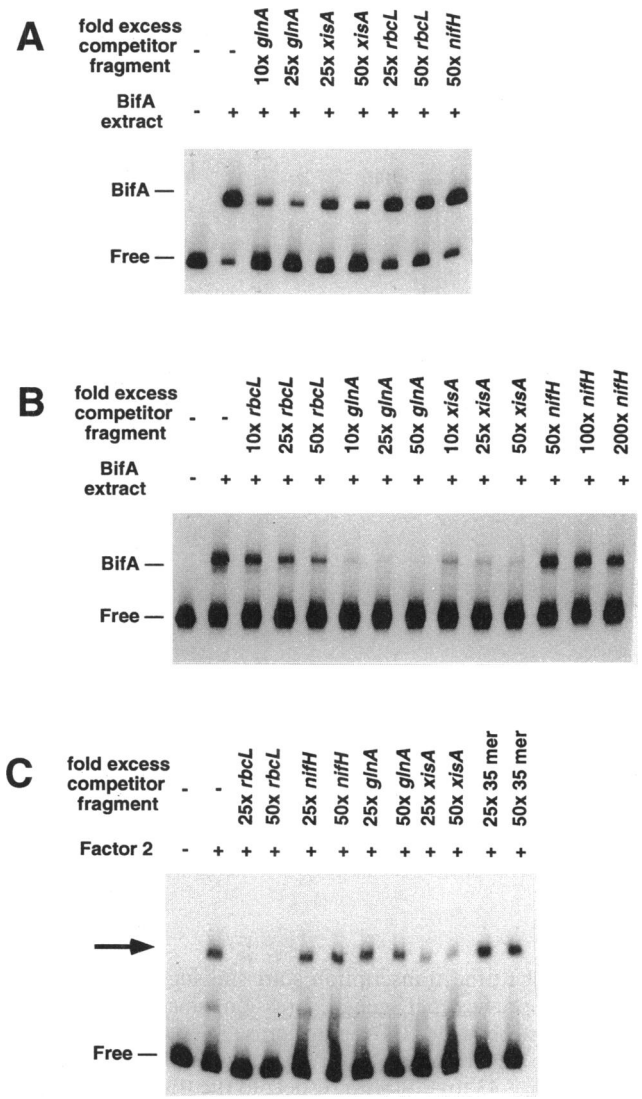

FIG. 4. Competition of *glnA*, *xisA*, *rbcL*, and *nifH* promoter fragments for BifA and factor 2 binding to labeled *glnA* or *rbcL* probe. (A) Labeled *glnA* probe binding to BifA. (B) Labeled *rbcL* probe binding to BifA. (C) Labeled *rbcL* probe binding to factor 2. Mobility shift assays were carried out using 5 μl (0.4 μg of total protein) of heparin-Sepharose column fractions containing either BifA or factor 2 and 6,000 cpm of the labeled DNA fragment with or without unlabeled competitor DNA fragment. Fractions were collected during elution with a linear 0.1 to 0.5 M ammonium sulfate gradient. The labeled DNA fragment was an *Xba*I-*Hin*dIII fragment from pAM658 (specific activity of 1.2×10^7 cpm μg^{-1}) containing 249 bp of *glnA* upstream region or an *Xba*I-*Hin*fI fragment from pAM496 (specific activity of 2.1×10^7 cpm μg^{-1}) containing 275 bp of *rbcL* upstream region. The competitor fragments used were an unlabeled *glnA* fragment, an *rbcL* fragment, a 235-bp *xisA* fragment from pAM709, a 191-bp *nifH* fragment from pAM657, and a 35-bp oligonucleotide (35-mer) that contains the BifA binding site on *glnA*. The fold excess unlabeled competitor fragment added, on a molar basis, is indicated above the lanes. The protein-DNA complex was run on a 5% polyacrylamide gel at 30 mA for 3 h. The positions of free DNA probe and the BifA-DNA complex are shown; the arrow marks the position of the factor 2-DNA complex.

protected region, which flanks the transcription start site, showed two DNase I-hypersensitive sites. In addition to the obvious protected regions, a DNase I-hypersensitive site and protection of a single base were seen around −480 bp. Although the distal DNase I-protected region extends to the bottom of the gel shown in Fig. 5A, other gels run for a shorter time confirmed the extent of the region protected (data not shown).

Factor 2 binding sites on *rbcL* and *xisA*. DNase I footprinting with factor 2 identified a 63-bp protected region on *rbcL* (Fig. 5B). The binding site covers a region from −519 to −581 with respect to the translation start site, which places it 15 bp upstream of the transcription start site (30). DNase I-hypersensitive sites were detected at nucleotides −539 and −551. An identical region was protected from DNase I when the longer *rbcL* fragment AM496Δ1 was used (data not shown).

DNase I footprinting with factor 2 on the *xisA* upstream region showed a protected region of at least 68 bp from about −156 to −223 bp upstream of the translation start site (Fig. 5B). The binding site for factor 2 on the *xisA* upstream region shows considerable overlap with the BifA binding sites, which extend from nucleotides −152 to −205 (11). This result is consistent with our mobility shift assays using the *xisA* fragment (Fig. 3). No synergism or interference between BifA and factor 2 was observed in mobility shift experiments with the *xisA* upstream region (data not shown).

DISCUSSION

Heparin-Sepharose column chromatography has been used to resolve two DNA-binding activities present in PCC 7120 vegetative cell extracts. The DNA-binding protein BifA binds the *glnA*, *xisA*, *rbcL*, and *nifH* upstream regions (11), and the newly identified DNA-binding activity, factor 2, binds the *rbcL* and *xisA* upstream regions. BifA, which is present in both vegetative cells and heterocysts, binds the four DNA fragments with affinities that follow the order *glnA* > *xisA* > *rbcL* >> *nifH*. Factor 2, which is present only in vegetative cell extracts, binds strongly to a region upstream of the *rbcL* promoter and also binds to a region upstream of *xisA* that overlaps the three BifA binding sites.

DNase I footprinting of BifA to *glnA*, *rbcL*, and *xisA* shows a single 24-bp protected region on *glnA* and two protected regions of 12 and 24 bp on *rbcL* (this work) and a 54-bp protected region on *xisA* that contains three binding sites (11). The sequence 5′ ACATT 3′ was identified as a possible consensus BifA recognition sequence in the *xisA* upstream region (11). Comparison of the BifA binding sites on *glnA*, *rbcL*, and *xisA* allows us to extend and modify the consensus sequence. These binding sites can be aligned to produce the consensus sequence 5′ TGT($N_{9\ or\ 10}$)ACA 3′ (Fig. 6). This motif is similar to CRP and NifA recognition sequences (4, 28, 29). The BifA recognition sequence is present once in *glnA*, twice in *rbcL*, and three times in *xisA* (Fig. 7).

Because of the weak binding of BifA to the *nifH* promoter fragment, we have not been able to identify the binding site by DNase I footprinting. BifA binding to a labeled *nifH* promoter fragment was clearly seen in heparin-Sepharose-purified protein fractions (Fig. 1), but competition experiments showed that the in vitro BifA binding affinity for the *nifH* promoter fragment was much less than that for other fragments. The 170-bp *nifH* region that binds BifA does not contain an obvious TGT($N_{9\ or\ 10}$)ACA motif.

The *bifA* gene has been recently cloned by using the *glnA* binding site in an in vivo genetic selection procedure. The predicted BifA protein shows significant sequence similarity to

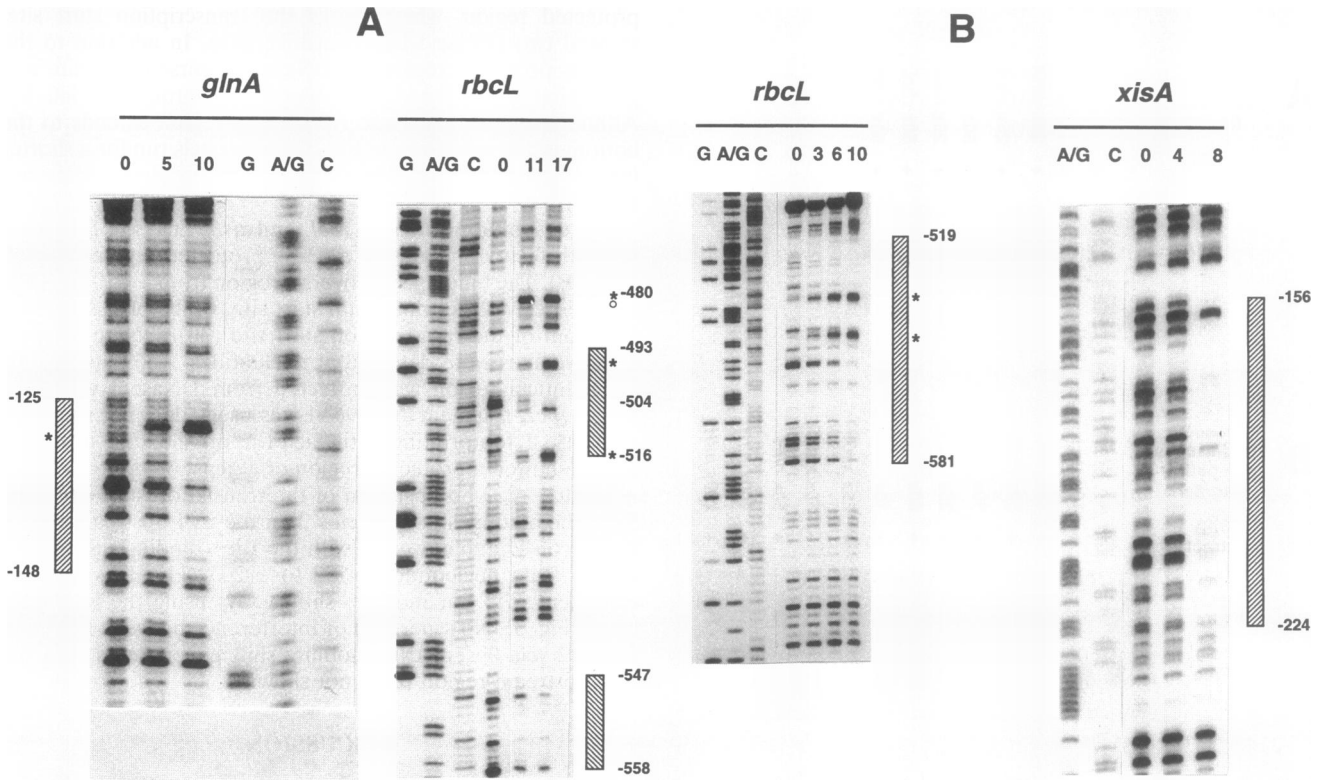

FIG. 5. BifA and factor 2 DNA-binding sites. (A) DNase I footprint of BifA on *glnA* and *rbcL* promoter regions. (B) DNase I footprint of factor 2 on *rbcL* and *xisA* upstream regions. The labeled DNA fragments were incubated with the indicated amounts (microliters of heparin-Sepharose column fractions containing 0.1 μg of total protein μl^{-1}) of either BifA or factor 2. Lanes G, A/G, and C are Maxam-Gilbert sequencing ladders produced from the labeled fragment. The Maxam-Gilbert sequencing ladders for *rbcL* in panel B used a fragment that was longer at the unlabeled end than the fragment used for DNase I protection. The hatched boxes mark the DNase I-protected regions. The nucleotide numbers are with respect to the translation start site. The asterisks indicate DNase I-hypersensitive sites, and the open circle indicates DNase I protection. In all cases the antisense strand was labeled.

a set of prokaryotic regulatory proteins represented by the *E. coli* CRP protein (39). Although BifA interacts weakly with the *nifH* promoter region and its recognition sequence is similar to that of NifA, it does not show significant amino acid sequence similarity to NifA (20, 39). CRP, FNR, and AraC are regulatory proteins that can act as both transcription activators and repressors (12). Depending on the position of its binding site with respect to different promoters, BifA may possibly act as an activator or a repressor of gene expression in PCC 7120.

DNase I footprinting of the *rbcL* promoter region with BifA showed the presence of two BifA binding sites (Fig. 7). The proximal 24-bp protected region from position +12 to −12 with respect to the transcription start site suggests that BifA probably represses *rbcL* gene expression, since BifA binding would interfere with RNA polymerase binding to the promoter. The significance of BifA binding to the distal 12-bp region from −43 to −54 with respect to the transcription start site, a position typical of activator proteins (12), is less clear. The BifA binding site on *glnA* maps to a region 35 nucleotides upstream of the major *nif*-like promoter (RNA_I) and 7 nucleotides downstream of the major *E. coli*-like promoter (RNA_{II}) (35). The position of the binding site would equally suit BifA as an activator of transcription of the *nif*-like promoter or as a repressor of transcription of the *E. coli*-like promoter. The *xisA* promoter has not been mapped, and therefore we do not know the relative positions of the BifA binding sites with respect to the transcription start site.

glnA	-142 **TGT**AACAAAGACT**ACA**	-123
rbcL	-510 **TGT**GAGACAAGTT**ACA**	-495
rbcL	-559 **TGT**AAGTTAAG.**AACT**	-546
xisA	-203 **TGT**TTG.CACTGAG**CA**	-189
xisA	-187 **TGT**TAG.TGATGA**ACA**	-173
xisA	-171 **TGT**TTGCTGAT.**AACA**	-155
Consensus	**TGT**–N(9 or 10)–**ACA**	

FIG. 6. Alignment of BifA binding sequences. The BifA binding sites on *glnA*, *rbcL*, and *xisA* are aligned 5′ to 3′ with respect to the open reading frame; numbers indicate the first and last nucleotides in the sequence and are relative to the translation start site set as +1. A consensus BifA binding sequence is shown.

The BifA binding sites on *rbcL* and *glnA* contain DNase I-hypersensitive sites (Fig. 7) which indicate perturbation of the DNA backbone. This suggests that BifA, like CRP, an archetypal DNA-bending protein (15), might cause bending of the DNA. However, the BifA binding site on *xisA* does not show any DNase I-hypersensitive regions on either strand (11).

Factor 2, which elutes earlier off of heparin-Sepharose columns than does BifA, is distinct from BifA in that it binds only to *rbcL* and *xisA* and not to *glnA* or *nifH*. Moreover factor 2's DNA-binding activity, unlike that of BifA, is absent from heterocyst extracts. A *bifA* mutant strain which lacks BifA DNA-binding activity (38) contains factor 2 activity. These

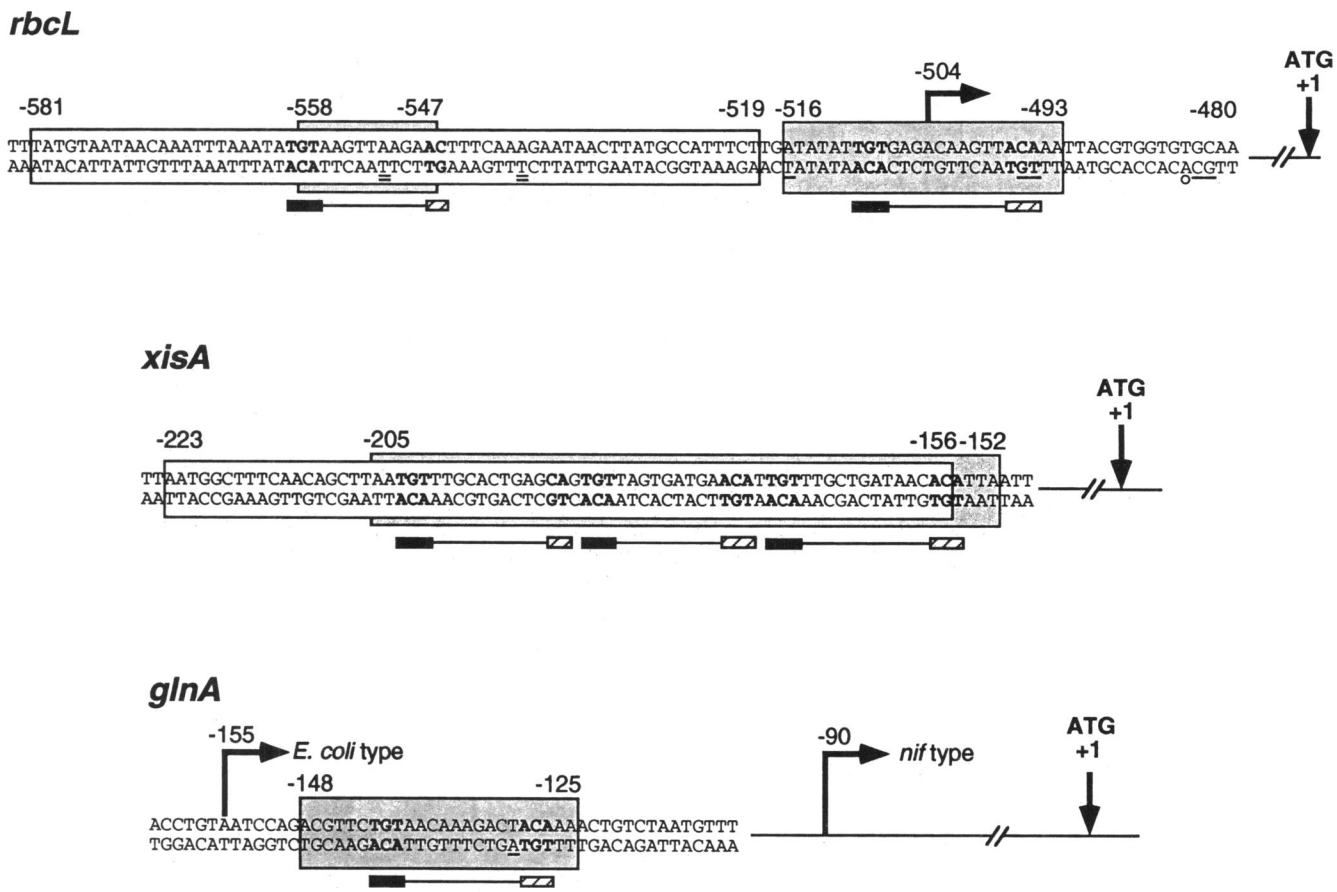

FIG. 7. BifA and factor 2 binding sites. The BifA binding sites on *rbcL*, *xisA*, and *glnA* upstream sequences are shown by gray boxes, and the factor 2 binding sites on *rbcL* and *xisA* are shown by open boxes. The consensus binding sequence for BifA, TGT($N_{9\ or\ 10}$)ACA, is shown in boldface and is marked below the sequence; the black rectangular box indicates the highly conserved sequence TGT, and the hatched rectangular box indicates the conserved sequence ACA. The DNase I-hypersensitive sites are underlined for BifA and double underlined for factor 2. The single BifA protected base at −482 in *rbcL* is indicated by an open circle. The horizontal arrows indicate transcription start sites, and the vertical arrows indicate translation start sites. The nucleotide numbers start with the A of the translation start site as +1. The major *E. coli*-type and *nif*-type transcription start sites for *glnA* are shown.

results show that factor 2 is a new PCC 7120 DNA-binding factor that interacts with genes that are differentially expressed in vegetative cells and heterocysts.

Comparison of the factor 2 binding sites in the *rbcL* and *xisA* upstream regions showed that the sequence 5′ CTTTCAA 3′ is common to both (Fig. 7). While a 235-bp *xisA* fragment binds both BifA and factor 2, a shorter, 206-bp *xisA* fragment binds only BifA. The sequence 5′ CTTTCAA 3′ is within the 29-bp sequence that is present in the 235-bp *xisA* fragment but absent from the 206-bp fragment, suggesting that it may be important for the interaction with the second DNA-binding activity. However, the putative consensus sequence is present in the middle of the binding site on the *rbcL* upstream region and towards the 5′ end of the binding site on the *xisA* upstream region. The significance of this sequence in binding site recognition by factor 2 is therefore questionable.

Our in vitro studies show that BifA and factor 2 are sequence specific DNA-binding proteins that interact with both vegetative cell- and heterocyst-specific genes, and they provide the basis for further studies. Our results suggest possible in vivo roles for BifA and factor 2 in PCC 7120. The *bifA* gene product shows 77% amino acid sequence identity to the cyanobacterial regulatory protein, NtcA, identified in *Synechococcus* sp. strain PCC 7942 (14, 36, 37, 39). NtcA is proposed to be a transcription activator required for full expression of genes subject to ammonium repression in PCC 7942. The consensus PCC 7120 BifA binding site is similar to the proposed NtcA binding site upstream of the PCC 7942 *glnA* and *ntcA* genes (14). BifA's interaction with *glnA* is consistent with it being homologous to NtcA, but BifA's interaction with *rbcL* does not fit the proposed function for NtcA. We propose that BifA can act as both a positive and a negative transcriptional regulator, as do other members of the CRP family (3, 31), and that its regulatory role in PCC 7120 may involve nitrogen metabolism, as well as other functions.

Whereas BifA DNA-binding activity is present in both vegetative cells and heterocysts, factor 2 DNA-binding activity is absent from heterocyst extracts. Since we do not know if factor 2 is a single protein or a complex of two or more proteins, changes in DNA-binding activity could result from changes that affect all or only some of its components. The reduced level of binding activity in heterocysts could be due to the absence of factor 2 or to a posttranslational modification of a protein such that it no longer binds DNA. It is possible that binding activity could be degraded in the mature heterocyst, since differentiating vegetative cells show enhanced proteolytic

activity (27). The presence of factor 2 activity primarily in vegetative cells and its ability to bind the vegetative cell-specific *rbcL* promoter in vitro would suggest that factor 2 is a positive transcriptional regulator of the *rbcLS* operon. The binding site for factor 2 on the *rbcL* promoter fragment is centered 47 bp upstream of the transcription start site, a position typical of activator proteins (12). The role of factor 2 as an activator protein, however, does not account for its in vitro interaction with the *xisA* upstream region, since *xisA* should not be expressed in vegetative cells. Determination of the significance of the interaction of factor 2 with *rbcL* and *xisA* will require cloning of the gene(s) encoding the DNA-binding activity and subsequent genetic studies.

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